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(54) Title: ADENOVIRAL VECTORS

(57) Abstract: The invention relates to adenoviral vectors comprising a mammalian regulatory region such as a cytokine regulatory region and a reporter gene operatively linked to the cytokine promoter. Preferably, the cytokine promoter is a TNFα promoter or an IL-10 promoter. The application also disclosesmethods of studying cytokine promoter activity and to cells containing the adenoviral vectors.

ADENOVIRAL VECTORS

The invention relates to adenoviral vectors, and in particular to adenoviral vectors comprising a mammalian regulatory region, such as a promoter, and a reporter gene operatively linked to the cytokine promoter. The invention also relates to cells containing the adenoviral vectors and to methods of using the adenoviral vectors.

Adenoviral vectors which are capable of carrying foreign DNA into cells are known in the art.

Adenoviruses exist as non-enveloped double-stranded DNA viruses. The adenovirus enters cells by a receptor-mediated endocytosis pathway. In the initial virus-receptor interaction, the adenovirus binds specific receptors present on the cell surface via fibres on its outer surface. Following attachment, the receptors with bound adenovirus cluster in coated pits, the virus is internalised within a clathrin-coated vesicle and, subsequently, into an endosomal vesicle, known as a receptosome or endosome. The adenovirus is ultimately transported to the nucleus where it directs synthesis of new nucleic acid and hence virus.

Adenovirus has been used as means of transporting DNA into cells. There are two means by which such transfer has been affected. Firstly, the adenovirus has been employed to facilitate the transfer of non-viral DNA either linked to the surface of the adenovirus or where the molecule is internalised, taken along within the adenoviral receptor-endosome complex. Secondly, the adenovirus has been employed to transfer non-viral molecules which are packaged within the adenovirus either in place of, or in addition to normal adenoviral genome. All systems have been used to take in exogenous nucleic acid placed within the double-stranded DNA genome of the virus. Such exogenous DNA has been placed under the control of a promoter to allow the exogenous nucleic acid to be transcribed.

The inventors have now produced adenoviral vectors comprising a mammalian regulatory sequence such as a cytokine promoter. Cytokines are peptide regulators of inflammatory and immune reactions. Substances considered as cytokines include, for example, interleukins 1-18, interferons, tumour necrosis factors (TNF), transforming growth factors (TGF),

platelet-derived growth factors, chemokines and colony-stimulating factors. Some compounds were sometimes previously known by the term "lymphokine".

Cytokines act in a complex interacting network on leucocytes, vascular endothelial cells, mast cells, haemopoietic stem cells, fibroblasts and osteoclasts, controlling proliferation, differentiation and/or activation through autocrine or paracrine mechanisms. Several cytokines, for example TNF-α and IL-1 are important inflammatory mediators and have been implicated in several chronic inflammatory and autoimmune diseases such as rheumatoid arthritis. The cytokines are being increasingly studied because of their importance in the formation of inflammatory and autoimmune diseases. Because the cytokines are involved in complex interconnecting reactions it is difficult to investigate the control of regulatory regions such as promoters or enhancers associated with the cytokines. The inventors realised that a cytokine promoter could be used to control a readily detectable reporter gene and allow the promoter to be studied. Furthermore, they recognised that adenovirus is a suitable vector because it enables the promoter-reporter gene construct to be inserted into a wide variety of cells including cultured cell-lines, normal cells such as macrophages, diseased tissue and primary cell-lines. The ability to insert a construct into primary cells is of particular importance because it allows the control of the promoter to be studied within, for example, diseased cells.

The use of adenovirus also had the unexpected benefit of allowing unexpectedly high levels of regulatory region-controlled reporter gene function and behaviour to be observed that were similar to the behaviour of the endogenous gene. These levels were far in excess of levels observed with other methods of inserting a cytokine regulatory region-reporter gene construct into cells. Furthermore, it has the advantage that, for the first time, cytokine promoters, such as TNF-α promoters, have been able to be studied in monocytic cells for the first time. It is expected that the high levels of expression observed means that such promoters can be studied in a variety of cells.

TNFα promoter-luciferase vectors have been produced by Udalova *et al* (J. Biol. Chem., 1998, Vol. 273, pages 21178-21186). However, the vector containing the construct, pXP-1,

can only be used in a limited number of cell types, and with low levels of expression of the promoter.

The invention provides an adenoviral vector comprising:

a) a mammalian regulatory region; and

b) a reporter gene operatively linked to the cytokine regulatory region (a).

The term "mammalian regulatory region" is intended to mean a region of DNA which, in its natural state, is upstream and/or downstream of a gene which it regulates. For example, it may be the promoter for the gene encoding a human oestrogen receptor.

The regulatory region may be a promoter or an enhancer region or a fragment of a region, such as an NF-κB binding site.

The regulatory region may regulate the expression of a cytokine gene. Preferably the cytokine promoter is an interleukin promoter such as IL-10. Preferably the IL-10 promoter has the sequence from NT 7 to NT 4046 of Genbank sequence number X78437, where NT 4023 is the transcription start site, as shown in Annex 2.

Alternatively the promoter may be derived from other genes that have application in inflammatory disease, such as IL-6 and MMP.

The cytokine promoter may also be the promoter for TNFα. More especially, the TNFα receptor is a human TNFα receptor, especially the TNFα promoter identified in Genbank Accession No. M16441. This is enclosed as Annex 1. This is also the promoter used in the Udilova paper (Supra). Most preferably the sequence used is encompassed within -1173 to +130 of the human TNF gene, where 1 is the transcriptional start site. The transcriptional start site is nucleotide 4094 in the Genbank Accession No. M16441. Hence, preferably the promoter sequence starts from 2921 of M16441.

Any suitable adenoviral vector can be utilised in the present invention. A "vector" is a molecule that serves to transfer nucleic acid of interest into a cell. Thus, the adenoviral

vector utilised in accordance with the present invention can encompass any adenoviral vector that is appropriate for the introduction of nucleic acids into eukaryotic cells and is capable of functioning as a vector as that term is understood by those of ordinary skill in the art.

The adenovirus can be any serotype of adenovirus and, preferably, is a serotype that can transduce and/or infect a human cell. The adenovirus may comprise a complete adenoviral virion consisting of a core of nucleic acid and a protein capsid. Alternatively, it may comprise a naked adenoviral genome or any other manifestation of adenovirus as described in the art which can be used to transfer a cytokine regulatory region operatively linked to a reporter gene. Alternatively the virion may contain minimal (packaging signal) adenoviral genome sequences. These "gutless" viruses are produced by methods known in the art.

The adenovirus employed for transfer of the regulatory region-reporter gene construct can be wildtype, that is replication-competent. However, it is not necessary that the genome of the employed adenovirus be intact. Indeed, the adenovirus can be rendered replication-deficient, by techniques known in the art.

The reporter gene is operatively linked to the mammalian regulatory region. By this we mean that the nucleic acid encoding the reporting gene is linked to the regulatory region in such a way that the regulatory region is capable of directing transcription of the reporter gene. The reporter gene may be any nucleic acid sequence encoding a detectable gene product. The gene product may be an untranslated RNA product, such as mRNA or antisense RNA. Such untranslated RNA may be detected by techniques known in the art such as PCR, Northern or Southern blots. Alternatively, the reporter gene may encode a polypeptide, such as protein or peptide, product. Polypeptide may be detected immunologically or by means of its biological activity. The reporter genes used may be any known in the art.

Preferably, the reporter gene is luciferase. Luciferase reporter genes are known in the art. They are usually derived from firefly (*Photinus pyralis*) or seapansy (*Renilla reniformis*). The luciferase enzyme catalyses a reaction using D-luciferin and ATP in the presence of oxygen and Mg^{2+} resulting in light emission. The luciferase reaction is quantitated using a

luminometer which measures light output. The assay may also include coenzyme A in reaction which provides a longer, sustained light reaction with greater sensitivity.

An alternative reporter gene to use is CAT (chloramphenicol acetyltransferase). This is a reporter gene which is well known in the art. CAT catalyses the transfer of the acetyl group from acetyl-CoA to the substrate chloramphenicol. The enzyme reaction can be quantitated by incubating cells or cell lysates with [^{14}C] chloramphenicol and following product formation by physical separation with, for example, thin layer chromatography or organic extraction. Alternatively, the CAT protein can be quantitated using an enzyme-linked immunosorbent assay. Such an assay is available from, for example, Promega Corporation, Southampton, United Kingdom.

A further reporter system which may be used is *lacZ* gene from *E.coli*. This encodes the β-galactosidase enzyme. This catalyses the hydrolysis of β-galactoside sugars such as lactose. The enzymatic activity in cell extracts can be assayed with various specialised substrates, for example x-gal, which allow enzyme activity quantitation using a spectrophotometer, fluorometer or a luminometer.

The reporter gene may also be gfp (green fluorescent protein) or β-globin, which are also known in the art.

The adenoviral vector preferably includes suitable termination sequences known in the art to allow the transcription of the reporter gene to be successfully terminated. Suitably, the vector also comprises a polyadenylation sequence. Such sequences may be derived from the gene from which the regulatory region is derived or alternatively from other sources such as an SV40 late polyadenylation signal to allow secretion of the reporter gene product. The termination sequences and/or polyadenylation sequences may be operatively linked 3' to the reporter gene.

The adenoviral vector may additionally comprise, 3' to the reporter gene, a 3'-UTR (untranslated region). Preferably, the 3'-UTR is from a cytokine. Preferably, the 3'-UTR is from the human TNF gene. In particular, the 3'-UTR may be from sequence identified as

Genbank Accession No. M16441, especially the sequences encoding 1041NT to the 3' of NT6071 of M16441. The sequences referred to in the paper by Udalova *et al* (Supra). 3'-UTR regions are thought to be involved effecting the stability and translation of mRNA. It is therefore advantageous to incorporate a 3'-UTR region to enable the effect of the region to be studied.

The adenoviral vector may additionally comprise one or more sequences to enable it to be maintained or replicated within a suitable bacterial host vector, such as *Esherichia coli.* Such sequences are well known in the art and include, for example, a bacterial origin or replication such as f1 ori. It may also include one or more genes to enable selection of bacteria comprising the adenoviral vector, such as ampicillin or tetracycline resistance genes known in the art.

The vector may additionally comprise a further reporter gene under the control of a constuitive or unregulated promoter such as CMV promoter. The further reporter gene will be different to the first reporter gene. It acts as an internal control, allowing variations, such as the amount of vector within each cell, to be taken into account.

A further aspect of the invention provides a method of studying mammalian regulatory region activity comprising inserting an adenoviral vector according to the invention into a cell and measuring the amount of reporter gene product produced by the mammalian regulatory region encoded by the adenoviral vector. The cell into which the vector has been inserted may be exposed to one or more inhibitors or activators of the mammalian regulatory region. Cells may or may not be activated with a stimulus such as lipopolysaccharide

Alternatively, the activity in a diseased cell, for example from rheumatoid arthritic joints, may be compared with the activity of the regulatory region within a non-diseased cell. This allows a better understanding of the diseased state to be studied.

The cell into which the adenoviral vector is inserted may be selected from normal cells, macrophages. dendritic cells, phagocytes, epithelium, endothelium, tissue or cell lines Adenoviral vectors of the invention can be introduced into cells using the natural capability of

the viruses and centre cells and to mediate uptake of macromolecules, that is by receptor-mediator endocytosis. Alternatively, the vectors can be introduced by any other suitable means such as transfection, calcium phosphate-mediated transformation, microinjection, electroporation, osmotic shock and the like.

The invention also provides a mammalian cell, especially a human cell, containing an adenoviral vector according to the invention. The type of cell may be selected from those listed above.

The invention also relates to kits comprising vectors according to the invention. Such kits may also include instructions for the use of the vector.

The invention also provides a method of screening a compound as an enhancer or an inhibitor of a mammalian regulatory region, comprising the use of an adenoviral vector according to the invention. the vector may be placed into a suitable cell, and the effect of the compound on the amount of reporter gene product produced observed. Preferably, the compound may act directly on the regulatory region or indirectly via, for example, a cascade pathway.

Embodiments of the invention will now be described by way of example only with reference to the following figures and examples.

Figure 1: Schematic representation of TNF reporter constructs.

The TNF promoter lying before the 5'UTR of TNF is represented as a thin line. The 5' and 3'UTR are represented by hatched bars. The luciferase coding sequence is shown as a solid bar. Locations of the start codon, stop codon and TATA box are indicated for each construct. For the construct TNF promoter 5'UTR, SV40 poly(A) sequence is represented by an open bar. For the construct TNF promoter 5'3'UTR, the AU-rich element (ARE) sequences are indicated as a grey box. Both constructs are produced in vector pGL3 and pAdeasy1 for transfection and adenovirus infection experiments, respectively.

Figure 2: Activity of TNF based reporter constructs when transfected or infected into RAW 264.7 and human macrophages.

A, B - cells were either transfected (A) or infected with adenoviral constructs at m.o.i. 40:1 (A, B). Following LPS (10 ng/ml.) stimulation (4h), cells were then harvested and assayed for luciferase. *C, D* - Induction by LPS (10 ng/ml.) or Zymosan (30 μg/ml.) of luciferase activity (C) and TNF production (D), in Advp5' and Advp5'3'UTR infected human macrophages. Cells were stimulated for 4h before assay. In absence of stimuli, TNF production was undetectable. Each experiment was repeated at least three times.

Figure 3: Comparison of the kinetics of LPS-induced TNF production and luciferase expression in human macrophages.

Cells were either uninfected (A) or infected with Advp5' (B) or Advp5'3'UTR (C) and either untreated (O). or activated with 10 ng/ml. LPS (●). At the indicated times, cell tysates or culture supernatants were harvested to assay luciferase or TNF production respectively. In the absence of LPS, TNF production was undetectable. Figure 3(D) Cells were infected with Advp5' (□) or Advp5'3'UTR (○) and activated with LPS for four hours. Actinomycin D was added to stop any further mRNA synthesis and the cells incubated for a further 0, 15, 30, 45 or 60 minutes. after which time they were harvested for RPA analysis of luciferase or GAPDH mRNA. The results are shown normalised to 100% at the 0 minute point. Each experiment was repeated at least two (D) or three (A-C) times with blood from distinct donors.

Figure 4: IL-10 inhibition of reporter gene constructs requires the 3'UTR.

Cells were infected with Advp5' (A) or Advp5'3'UTR (B) and activated simultaneously with LPS (10 ng/ml.) and various concentrations of IL-10. Luciferase activities (●) and TNF production (○) were assayed after 4h LPS stimulation. Ordinates represent the percentages of luciferase activation and TNF production induced by LPS, in the absence of IL-10. Data are mean values ± S.E.M. from 4 separate experiments conducted with blood from diffeerent donors.

Figure 5: Kinetics of IL-10 inhibitory activity on TNF production and luciferase expression.

Human monocytes-derived macrophages were uninfected (A) or infected with Advp5'3'UTR (B), or Advp5' (C) (m.o.i. 40:1) and then incubated with LPS in the presence (○), or absence (●) of IL-10 (10 ng/ml.). The experiment was harvested at indicated times and TNF and luciferase assayed. Data are mean values (±) from duplicates and are representative of three separate experiments conducted with distinct donors.

Figure 6: Effect of IL-10 on TNF and Luciferase mRNA levels.

Human macrophages infected with either Advp5', Advp5'3'UTR or Adv0 were treated with IL-10 (10 ng/ml.) simultaneously or 24h before LPS. Four hours after LPS activation, the experiment was harvested and ribonuclease protection assays were performed to quantify luciferase or TNF/GAPDH mRNA ratios. These are plotted as percentage of the maximum value in presence of LPS alone.

Figure 7: IL-10 can inhibit TNF expression even when added post-LPS stimulation.

Human macrophages infected with either Advp5' or Advp5'3'UTR (m.o.i. 40:1) were incubated for 4h in the presence of LPS (10 ng/ml.). IL-10 (10 ng/ml.) was added at the given times after LPS. Ordinates represent the percentages of luciferase activation and TNF production induced by LPS in the absence of IL-10. Data are mean values (±) from duplicates and are representative of three separate experiments conducted with distinct donors.

Figure 8: Effect of prolonged exposure to IL-10 prior to LPS stimulation on luciferase expression.

Human macrophages infected with either Advp5' or Advp5'3'UTR were treated with IL-10 (10 ng/ml.) for the given times prior to LPS-stimulation. Four hours after LPS activation, the experiment was harvested and luciferase activities and TNF expression assayed. Data are mean values (±) from duplicates and are representative of three separate experiments conducted with distinct donors.

Figure 9: Prolonged exposure to IL-10 prior to LPS-stimulation results in the inhibition of luciferase expression in the absence of 3'UTR.

Infected cells (Advp5' (A), Advp5'3'UTR (B) were pre-treated for 24h with various concentrations of IL-10 before LPS exposure. The experiment was harvested after 4h LPS stimulation and luciferase activities (●) and TNF production (○) were assayed. Data are expressed as percentages of luciferase activation and TNF production induced by LPS in absence of IL-10. Data are mean values (± SEM) from four experiments done with different donors.

Figure 10: In murine cells, IL-10 inhibition of luciferase expression requires the 3'UTR regardless of length of exposure to the cytokine.

Infected cells (m.o.i. 40:1) were incubated in presence of LPS (10 ng/ml.) without (■), or with IL-10 (10 ng/ml.) added simultaneously (□) or 24h before LPS addition (□). After LPS stimulation (4h), mTNF production and luciferase activities were assayed. Ordinates represent the percentages of luciferase activation and TNF production induced by LPS in the absence of cytokine. The results are representative of three (RAW264.7 cells) or two (primary murine macrophages) experiments.

METHODS

Plasmids

Human *TNF* promoter (-1173bp) with 3'untranslated region of the human TNF gene (pGL3-*TNF*-3'UTR) or without the 3' UT region (pGL3-*TNF*) (Udalova *et al.*, 1998) and the human IL10 promoter construct (-4080bp) (pGL3-*IL10*) were subcloned into the pAdTrack vector (He *et al.*, 1998) to generate pAdTrack-*TNF*-Luc-3'UTR, pAdTrack-*TNR*-Luc and pAdTrack-*IL10*-Luc. For both later ones, KpnI/SalI fragments containing the human TNF or IL10 promoter, the luciferase reporter gene and the SV40 late poly (A) signal were derived from their pGL3 respective constructs and cloned into KpnI/SalI sites of AdTrack vector.

The 3'UTR construct was obtained by substituting a XbaI/BamHI fragment containing the SV40 late poly(A) signal in the pGL3-*TNF* plasmid for approximately 1kbp of 3'UTR amplified by PCR with corresponding primers: 3'UTR-F (XbaI): aattctagaGGAGGACGAACATCCAAC; 3'UTR-R(BamHI): aatGgATcCCCAGAGTTGGAAATTC. The KpnI/SalI fragments were subsequently cloned into the pAdTrack vector.

Adenoviral vectors and their propagation

The pAdEasy-1 adenoviral plasmid was provided by B. Vogelstein (The Howard Hughes Medical Institute, Baltimore, MD) and disclosed in He et al. Recombinant viruses were generated in BJ5183 bacterial cells transformed by the heat-shock method with 1μg of linearised modified AdTrack constructs and 100ng of replication-deficient adenoviral vector pAdEasy-1. Positive recombinant clones were selected through their resistance to kanamycin. Following selection DNA extracted was used for virus propagation in the 293 human embryonic kidney cells. Viruses were purified by ultracentrifugation through two cesium chloride gradients as described in He *et al.*, 1998. Titres of viral stocks were determined by plaque assay in 293 cells after exposure for 1 hour in serum free DMEM medium (Gibco BRL) and subsequently overlayed with 1.6% agarose/2xDMEM with 4% FCS mixture v/v1:1 and incubated for 10-14 days (He *et al.*, 1998).

Cells

Mononuclear cells were isolated from single donor plateletphoresis residues by Ficoll-Hypaque centrifugation proceeding monocyte separation in a Beckman JE6 elutriator (High Wycombe, UK). Monocyte purity was assessed by flow cytometry with CD14 and was approx. 80% (Williams *et al.*, 1996).

The elutriated human monocytes were incubated at 1 x 10^6/ml in RPMI 1640 with 25 mM HEPES and 2 mM L-glutamine supplemented with 10% (v/v) heat-inactivated FBS and 10 U/ml penicillin/streptavidin. To optimize infection, purified human monocytes were pretreated with M-CSF at 100ng/ml (Genetics Institute, Boston, MA) for 48h to allow up-regulation of integrin $\alpha_v\beta_5$, which has previously been shown to be essential for adenovirus infection of monocytes (Huang *et al.*, 1995).

RAW 264.7 mouse macrophages were maintained in DMEM supplemented with 10% FCS and 10 U/ml penicillin/streptavidin.

Transfection and Infection

0.5 x 10^6 RAW 264.7 cells/well were plated in growth medium (6 wells/plate). The following day, cells were transfected with 100 ng of DNA by using Superfect (Qiagen, Germany). For infection, these cells were plated at 0.1 x 10^6/well (48 wells/plate). After M-CSF treatment human macrophages were replated at 0.2 x 10^6/well (96 wells/plate). RAW 264.7 and M-CSF treated macrophages were then exposed to virus for 1h in serum free medium followed by washing and reculturing in growth medium with 2% FCS for 24h. For Adv *TNF*-Luc and Adv *TNF*-Luc-3'UTR, RAW 264.7 and M-CSF-treated macrophages were infected with a multiplicity of infection (MOI) of 40:1. Infected or transfected cells were then stimulated with LPS (10 ng/ml) for 4h. Infection of M-CSF treated macrophages by Adv *IL10*-Luc was efficient at MOI of 80:1 and cells were stimulated for 18h with LPS (*Salmonella typhimorium*, Sigma, Poole, UK.).

Luciferase assay

After LPS stimulation, cells were washed once in PBS and lysed with 100 µl of CAT lysis buffer (0.65% (v/v) of NP40, 10 mM of Tris-HCL pH 8, 0.1 mM EDTA pH8, 150 mM MaCl). 50 µl of cell lysate was transferred into the well of a luminometer cuvette strip and Luciferase Assay Buffer (220 µl) added. Luciferase activity was measured with a Labsystem luminometer by dispensing 30 µl luciferin (1.5 mM, Sigma) per assay point.

Measurement of hTNF and mTNF production.

TNF levels were measured in cell supernatants by sandwich enzyme-linked immunosorbant assay (ELISA). A polyclonal rabbit anti-mouse TNF antibody for coating and the same biotinylated polyclonal antibody were used to detect mTNF. ELISA for hTNF was performed as previously reported (Engelberts, I., *et al.* 1991, *Lymphokine Cytokine Res.* **1-2**, 69).

Ribonuclease protection assay (RPA).

After M-CSF treatment, cells were plated at 2 x 10^6/w in 12-well plate and infected as described above. In Advp5' and Advp5'3'UTR infected cells Luciferase and GAPDH mRNAs were detected by ribonuclease protection assay by using luciferase and GAPDH riboprobes, respectively. In parallel, TNF and GAPDH mRNAs were detected in Adv0-infected cells. Riboprobe vectors were constructed as follows. A 352-bp *HincII-XbaI* luciferase fragment was cloned from pGL3c (Promega) into pBluescript KS that had been digested with *EcoRV* and *XbaI*. A 268-bp TNF gene fragment was amplified by PCR from human genomic DNA, and subcloned into *SpeI* site of pBluescript KS^+ (kindly provided by Dr A Clark, Kennedy Institute, London, UK.) that had been digested with *SpeI*. Riboprobe template constructs were linearized by appropriate restriction and purified by phenol-chloroform extraction and ethanol precipitation. Luciferase and GAPDH riboprobes were synthesized using T7 polymerase and TNF riboprobe by using T3 polymerase (Boehringer Mannheim) in the presence of 50 µCi of [α-32P]UTP (800 mCi/mmol; Amersham). The final concentration of unlabelled UTP in *in vitro* transcription reactions was 12 µM, except in the case of Luciferase where it was 2.4 µM. Ribonuclease protection assays were carried out using the Direct

Protect kit (Ambion). Under the conditions of hybridization DNA-RNA heteroduplexes were not detected. Protected RNA fragments were resolved by electrophoresis on denaturing 6% polyacrylamide gels and were visualized and quantified by phosphorimaging (Fuji FLA 2000) and autoradiography. Each experiment was performed twice, and serial dilutions of lysates were used to check that quantitations were within the linear range of the assay.

RESULTS

Comparison of TNF reporter gene activity when delivered by adenoviral infection as opposed to standard DNA transfection.

The TNF gene reporter constructs used in this study are schematically illustrated in Figure 1. The 5'promoter and 5'promoter-3'UTR constructs, were incorporated into the pGL3 vector for transient transfection experiments (using Lipofectin), or recombinant adenoviruses (reporter viruses, Advp5' and Advp5'3'UTR respectively) for infection studies. Initial studies compared the response of the reporter gene to LPS when delivered by transfection, with infection. The RAW 264.7 murine macrophage cell line was used for these studies, as our attempts to transfect primary human monocytes or macrophages have proved unsuccessful. Lipofection was the transfection method of choice as, in our hands, this had been found to be the most efficient for the RAW 264.7 cells (results not shown). LPS stimulation of transfected cells gave a modest absolute signal nd resulted in an approximate 20-fold response by both 5' and 5'3' constructs. This level of response was not dissimilar to other reported studies with the same type of construct and technique in this cell line (Hacker *et al.* (1999) *Embo J.* 24, 6973). For comparison, both RAW 264.7 cells and primary human macrophages were infected with virus at m.o.i. 40:1, as previous studies had indicated that this concentration resulted in the successful infection of >90% of cells ((13, data not shown). In contrast to the transfection study, RAW 264.7 cells, infected with reporter adenovirus, gave LPS responses of 60-70 fold and much higher absolute values of luciferase activity (Figure 2A). LPS activation of the reporter gene in human macrophages infected with the reporter viruses resulted in 166 and 115 fold stimulation of the 5' and 5'3'UTR constructs respectively (Figure 2B). The strength of response varied from donor to donor and it has not been uncommon to obtain activations in excess of 250 fold. The greater response of human compared to murine cells has been a consistent finding. One possible reason for this is that the reporter gene constructs are based on the human sequence. The reporter gene also responded equally well to the yeast product, zymosan, an alternative stimulus of TNF production with a similar potency to LPS (Figure 2C, 2D). A consistent finding in all systems, regardless of the means of gene delivery or stimulus, was the lower absolute response of constructs containing the 3'UTR. These data would support the view, obtained

from previous studies only in murine macrophage cell lines, that the 3'UTR is generally suppressive to TNF expression (Han (1991) *J. Immunol.* **6**, 1843). The powerful inducible response of the TNF gene is its notable characteristic. Therefore, it was interesting to find that the virally delivered reporter gene gave responses (activation in excess of 100 fold) that are much more comparable to the endogenous TNF gene than the transfected constructs (activation of 10 fold).

Infection

Cell type	Virus	Luciferase activity		Fold activation
		No LPS	With LPS	
RAW 264.7	Adv *TNF*-Luc	3 ± 2	216 ± 206	75 ± 34 (N=6)
	Adv *TNF*-Luc -3'UTR	0.8 ± 0.3	49 ± 60	51 ± 47 (N=6)
M-CSF treated human macrophage	Adv *TNF*-Luc	30 ± 26	4241 ± 1869	252 ± 165 (n=8)
	Adv *TNF*-Luc-3'UTR	7.5 ± 8.7	1008 ± 660	150 ± 86 (n=8)
	Adv *IL10*-Luc	1.8 ± 2	220 ± 96	159 ± 100 (n=5)

Transfection

Cell type	DNA	Luciferase activity		Fold activation
		No LPS	With LPS	
RAW 264.7	pGL3-*TNF*	2 ± 1	19 ± 7	10 ± 1 (n≥3)
	pGL3-*TNF*-3'UTR	0.17	2.5	15 (n≥3)

Kinetic studies were also performed to compare the behaviour of the reporter genes with the endogenous gene in human macrophages. In response to LPS, TNF production reached a maximum four hours after stimulation and thereafter, decreased slowly with a half-life of approximately 18.5 hours (Figure 3A). The kinetics of luciferase activity from Advp5' or Advp5'3'UTR infected LPS-stimulated human macrophages followed a similar profile to TNF, with maximum expression at 4 hours and apparent half-lives estimated to be 23.5 and 12 hours respectively (Figure 3B, 3C). The shorter half-life of luciferase, when under the addition control of the 3'UTR, might be expected from the overall destabilising effect of this element on TNF mRNA (Hacker, Supra; Kruys, *et al.* (1993) *J. Exp. Med.* **5**, 1383). To confirm this, studies on luciferase mRNF stability were performed that showed the presence

of the 3'UTR does indeed increase the rate of decay of luciferase mRNA (Figure 3D). In the presence of the 3'UTR the mRNA had a half life of 56 minutes, however, in the absence of this element, there was little decay (<15%) within the same time frame. One might have expected that the half-life of the endogenous TNF protein would have been comparable to the 5'3'UTR rather than the 5' construct. However, these data do not take into account potential differences in the biological half-lives of the TNF and luciferase proteins and one can only assume that the half-life of the endogenous TNF would be longer in the absence of the 3'UTR in humans, as shown previously in mouse cells with deletions of the 3'UTR on the AU-rich region of the 3'UTR.

In primary human macrophages, IL-10 requires the 3'UTR to inhibit TNF production.

The effectiveness of adenovirus delivery of TNF-based reporter gene constructs to primary human macrophages led us to investigate what role the 5' and 3' regions may play in the IL-10 inhibition of TNF expression. Adeno-reporter virus-infected cells were simultaneously treated with LPS and various concentrations of IL-10 for 4 hours, after which TNF production and luciferase activities were assayed (Figure 4). IL-10 inhibited TNF expression to a maximum of 80% at 10 ng/ml. (Figure 4A, 4B). However, the response of the two reporter constructs was quite distinct. The 5' construct was only weakly inhibited by IL-10, approximately 10%, whereas the 5'3'UTR construct showed a dose response profile similar to the endogenous TNF, although the maximum inhibition attained was less, 60% at 10 ng/ml. The IC_{50} for IL-10 on TNF protein was 0.2-0.3 ng/ml., compared with 2-3 ng/ml. for the reporter gene. However, if the concentration of half-maximal inhibition is calculated, then the activity of IL-10 is similar for the endogenous gene (~0.1 ng/ml.) and the 5'3'UTR construct (0.2-0.3 ng/ml.). This suggests that aspects of the inhibitory activity of IL-10 on the reporter gene and the endogenous gene are similar. The kinetics of the IL-10 inhibition of TNF and luciferase expression was also compared using the optimal concentration of 10 ng/ml. (Figure 5). Over the two to twenty-four hour period post-stimulation, the expression of TNF and the 5'3'UTR construct gave very similar profiles with very little response detected in the presence of IL-10 (Figure 5A, 5B). The effect of IL-10 on the 5' construct was, again, much weaker, with no significant inhibition over the time course (Figure 5C). These data would suggest that the major inhibitory effect of IL-10 is mediated via the 3'UTR and that

there appears to be little effect on the transcription of the gene when IL-10 is administered at the same time as LPS. To gain a further insight into the mechanism of IL-10 activity, luciferase mRNA levels were analysed by RPA. As shown in Figure 6, simultaneous IL-10 addition caused a marked reduction of luciferase mRNA from the 5'3'UTR construct, whereas, there was only a marginal effect on mRNA from the 5' construct. These data indicates that IL-10 causes a potential destabilization/enhanced destruction of mRNA via the 3'UTR. Data obtained with TNF mRNA showed identical results to the 5'3'UTR construct (Figure 6).

The data so far indicates that IL-10 mediates its activity on the post-transcriptional mechanism associated with TNF production. If this is so, IL-10 should still be able to inhibit TNF production, at least for a period, if added after LPS. Reporter virus infected macrophages were stimulated with LPS and IL-10 was added for periods of up to 2 hours post-activation; as before the experiment was harvested at 4 hours for assay. As expected, IL-10 had little effect on the activity of the 5' construct, regardless of when it was added (Figure 7). The inhibitory effect of IL-10 on TNF and the expression of the 5'3'UTR construct was maintained, even if IL-10 was added 1 hour after LPS activation but was greatly reduced if the cytokine was added 2 hours post-activation (Figure 7). These data would suggest that IL-10 does not inhibit the early events that are involved in TNF production (e.g. transcription) and instead, targets later events (e.g. post-transcriptional). This agrees with the hypothesis that post-transcriptional control mediated via the 3'UTR is the target of IL-10.

Pre-incubation of human macrophages with IL-10 before LPS stimulation reveals a second mechanism of inhibiting TNF production through the 5' promoter.

As time of exposure to IL-10 could obviously have a bearing on its function, this study was extended to investigate the effect of adding IL-10 **before** LPS for periods up to 24 hours (Figure 8). Pre-incubating macrophages with IL-10 for 12 hours produced a modest increase in the inhibition of endogenous TNF production or luciferase activity from the 5'3'UTR reporter gene when compared with the effect of simultaneous addition of LPS and IL-10 (Figure 8). Further periods of pre-incubation up to 24 hours did not elicit any major

additional effect. However, pre-exposure of cells to IL-10 had a profound effect on the expression of the 5' reporter. Pre-incubation of macrophages with IL-10 for twelve hours before LPS stimulus resulted in 50% inhibition of the 5' construct that increased to nearly 70% when the pre-incubation period was extended to 24 hours (Figure 8). This was compared to the 80% inhibition of endogenous TNF production. The effect of pre-incubating macrophages for 24 hours with different concentrations of IL-10 was also examined. As shown in Figure 9, LPS-induced TNF protein expression was inhibited to a maximum of 90% (10 ng/ml. IL-10) with an IC50 of ~0.1 ng/ml. regardless of which reporter construct had been infected into the macrophages. IL-10 also inhibited the expression of the 5'3'UTR reporter to an identical degree to the endogenous gene (Figure 9B). However, in contrast to data in Figure 4A, pre-incubation for 24 hours with IL-10 now produced a dose-dependent inhibition of the 5'UTR construct that showed a maximum inhibition of 60% at 10 ng/ml. (Figure 9A). The IC50 for IL-10 on the 5' construct was 5 ng/ml. but this reduced to 0.5 ng/ml. if the half-maximal inhibition was again calculated. These data would indicate that in addition to post-transcriptional regulation of the TNF gene, IL-10 can also inhibit transcription of the TNF gene if cells are exposed to this inhibitory factor for a sufficient period. Indeed, studies on mRNA levels showed that pre-incubation, for 24 hours, with IL-10 resulted in a decrease in luciferase mRNA regardless of the presence of the 3'UTR (Figure 6).

IL-10 cannot inhibit TNF gene by the 5' promoter in primary murine macrophages and cell lines.

To the inventors' knowledge, the potential dual mechanism of IL-10 inhibitory activity on TNF expression has not been previously described. However, the type of reporter gene study described here is also novel. We therefore applied the adeno-reporter virus approach to murine macrophage cell lines and murine peritoneal derived macrophages systems that are more commonly used to investigate IL-10 function. We have previously shown that the murine macrophage cell line RAW 264.7 woulc be infected by adenovirus (Figure 2), and we have found that murine peritoneal derived macrophages were also permissive to virus infection (results not shown). We observed that a m.o.i. 40 of adenovirus was sufficient to infect murine primary cells and that LPS would activate both the 5' and 5'3'UTR constructs

(results not shown). Murine cells were infected with reporter viruses and were either pre-incubated with IL-10 for 24 hours or given the cytokine simultaneously with LPS (Figure 10). We had previously ascertained that 10 ng/ml. IL-10 was an optimal concentration for the inhibition of TNF production by the murine cells (23). For simplicity the data with IL-10 from each study are shown as a comparison with the LPS only control that is given as 100% (Figure 10). In RAW 264.7 cells, IL-10 inhibited TNF production and 5'3' luciferase construct to a similar degree when added with LPS. However, there was no inhibition of the 5' construct even when RAW 264.7 cells were pre-incubated with IL-10. Also, pre- exposure to the cytokine reduced the inhibition of luciferase expression by the 5'3' construct although the inhibition of endogenous TNF was maintained.. The study was repeated on primary murine macrophages since RAW 264.7 are transformed and this could affect the mechanisms of IL-10 action. IL-10 produced a profound inhibition of TNF production by primary murine macrophages regardless of length of treatment with cytokine. However, the effect on the expression of 5'3'UTR was only moderate, achieving a 30% inhibition. Again, this was not affected by pre-incubation with IL-10. There was no effect on the expression of the 5' construct under any of these circumstances. A trivial reason for the failure to demonstrate any effect on 5' construct, at least on the murine cells, was that these studies were performed with human rather than murine IL-10. However, repetition of these studies with the murine IL-10 gave similar results (results not shown).

DISCUSSION

The results show that viral transfer allows improved reporter gene induction (60 to 80 fold) in cell lines and even more so in primary cells (approximately 200 fold) following LPS stimulation. This result is unexpected and demonstrates that use of an adenoviral construct has unexpected advantages over the prior art.

The constructs have also been demonstrated to be able to be used to study regulatory region, such as cytokine, activity with primary macrophages which has previously been difficult to undertake.

REFERENCES

Udalova, I.A., Knight, J.C., Vidal, V., Nedospasov, S.A. & Kwiatkowski, D. Complex NF-kappaB interactions at the distal tumor necrosis factor promoter region in human monocytes. J Biol Chem **273**, 21178-86 (1998).

He, T.C., *et al.* A simplified system for generating recombinant adenoviruses, Proc Natl Acad Sci USA **95**, 2509-14 (1998).

Huang S., J. Virol., 69(4), 2257-63 (1995).

Williams, L.M., Gibbons, D.L., Gearing, A., Maini, R.N., Feldmann, M. & Brennan, F.M. Paradoxical effects of a synthetic metalloproteinase inhibitor that blocks both p55 and p75 TNF receptor shedding and TNFα processing in RA synovial membrane cell cultures. J Clin Invest **97**, 2833-41 (1996).

ANNEX 1

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SEQUENCE LENGTH (SQL):	7112
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DIVISION CODE (CI):	Primates
DATE (DATE):	14 Jan 1995
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KEYWORDS (ST):	lymphotoxin; tumor necrosis factor
SOURCE:	Human placenta DNA, clone pTNF186.
ORGANISM (ORGN):	Homo sapiens Eukaryotae; mitochondrial eukaryotes; Metazoa; Chordata; Vertebrata; Eutheria; Primates; Catarrhini; Hominidae; Homo
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ORIGIN:	1 bp upstream of EcoRI site; chromosome 6p21.3.
COMMENT:	Draft entry and computer-readable sequence for [1] kindly submitted by C.V.Jongeneel, 02-OCT-1988.
REFERENCE:	1 (bases 1 to 7112)
AUTHOR (AU):	Nedospasov,S.A.; Shakhov,A.N.; Turetskaya,R.L.; Mett,V.A.; Azizov,M.M.; Georgiev,G.P.; Korobko, V.G.; Dobrynin,V.N.; Filippov,S.A.; Bystrov,N.S.; Boldyreva,E.F.; Chuvpilo,S.A.; Chumakov,A.M.; Shingarova,L.N.; Ovchinnikov,Y.A.
TITLE (TI):	Tandem arrangement of genes coding for tumor necrosis factor (TNF-alpha) and lymphotoxin (TNF-beta) in the human genome
JOURNAL (SO):	Cold Spring Harb. Symp. Quant. Biol., 51 Pt 1, 611-624 (1986)
OTHER SOURCE (OS):	CA 107:110246

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ANNEX 2

GENBANK.RTM. COPYRIGHT 1999

```
LOCUS (LOC):              HSINTL10     GenBank (R)
GenBank ACC. NO. (GBN):    ***X78437***
CAS REGISTRY NO. (RN):    156795-75-4
SEQUENCE LENGTH (SQL):    4181
MOLECULE TYPE (CI):       DNA; linear
DIVISION CODE (CI):       Primates
DATE (DATE):              27 Jan 1997
DEFINITION (DEF):         H.sapiens IL-10 gene.
KEYWORDS (ST):            IL-10 gene; interleukin 10
SOURCE:                   human.
 ORGANISM (ORGN):         Homo sapiens
                          Eukaryotae; mitochondrial eukaryotes; Metazoa;
                          Chordata; Vertebrata; Eutheria; Primates;
                          Catarrhini;
                          Hominidae; Homo
NUCLEIC ACID COUNT (NA): 1181 a   1046 c   961 g   993 t
REFERENCE:                1  (bases 1 to 4181)
   AUTHOR (AU):           Kube,D.; Platzer,C.; von Knethen,A.; Straub,H.;
                          Bohlen,H.; Hafner,M.; Tesch,H.
   TITLE (TI):            Isolation of the human interleukin 10 promoter.
                          Characterization of the promoter activity in
Burkitt's
                          lymphoma cell lines
   JOURNAL (SO):          Cytokine, 7 (1), 1-7 (1995)
   OTHER SOURCE (OS):     CA 122:125051
REFERENCE:                2  (bases 1 to 4181)
   AUTHOR (AU):           Kube,D.
   TITLE (TI):            Direct Submission
   JOURNAL (SO):          Submitted (21-MAR-1994) D. Kube, Medizinische
Klinik I
                          der Universitaet zu Koeln, LFI Ebene 4 Raum 508,
                          Joseph-Stelzmann-Str 9, 50924 Koeln, FRG
REFERENCE:                3  (bases 1 to 4181)
   AUTHOR (AU):           Kube,D.
   TITLE (TI):            Direct Submission
   JOURNAL (SO):          Submitted (26-APR-1995) D. Kube, Medizinische
Klinik I
                          der Universitaet zu Koeln, LFI Ebene 4 Raum 508,
                          Joseph-Stelzmann-Str 9, 50924 Koeln, FRG
REFERENCE:                4  (bases 1 to 4181)
   AUTHOR (AU):           Kube,D.
   TITLE (TI):            Direct Submission
   JOURNAL (SO):          Submitted (25-JAN-1996) D. Kube, Medizinische
Klinik I
                          der Universitaet zu Koeln, LFI Ebene 4 Raum 508,
                          Joseph-Stelzmann-Str 9, 50924 Koeln, FRG
REFERENCE:                5  (bases 1 to 4181)
   AUTHOR (AU):           Eskdale,J.; Kube,D.; Gallagher,G.
   TITLE (TI):            A second polymorphic dinucleotide repeat in the 5'
                          flanking region of the human IL10 gene
   JOURNAL (SO):          Immunogenetics, 45 (1), 82-83 (1996)
   OTHER SOURCE (OS):     CA 126:113969

FEATURES (FEAT):
  Feature Key       Location                Qualifier
==============+=====================+================================
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/tissue-type="placenta"
/clone-lib="pWe15 cosmid library"

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CDS	4051..>4181	/gene="Il-10" /codon-start=1 /product="interleukin-10" /db-xref="PID:e220327" /db-xref="PID:g1177469" /translation="MHSSALLCCLVLLTGVRASP GQGTQSENSCTHFPGNLPNMLRDL "

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 121 gaaacttact gggcggagct tcctaaaaag attattaggg tctcctgggt tggtgtgcct
 181 ttaaaccttt ggactttacc acctcctatc tctcctatct ccttgcaaca aaggttagga
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 301 tgatgagtga gacagaaaat aaaatacaac ccctctcttt aaaagccatg cttactcagg
 361 ttttccttca tttgcagcta aatacagaaa tgagagaata ttttggagca gggatggaag
 421 aagagaggta ttccccttcc cacaaccttc tgatttccca gtacatcccc cactggaaaa
 481 attcatttaa aatcagtata ataagcattg attagatgcc tactatgcat ctgggcttga
 541 gggcaaactg gactcaggcc ttttggcctc aagaagctca cagtgtgaga gtggcatttg
 601 tgtcctcttg aaattcacag gactaaattg tgcccaggct gacattctat ccatccatag
 661 gtgcctgcct tctcacttcc ctctcttcat gggctcttgc cttgtaccaa aatccaaacc
 721 caaatctcct cacatgtgag tgttggcatt catgtctcag acatgaccta tgggcttggg
 781 acttttcccc gtggacccca gtgacttttc agatgaacag gtatcttcaa aaacttgaga
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1081 caaatcttca gcaaatggct tgagataatt agctacacat ttcagaacaa ataaagaagt
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1201 cgggcggatc ataaggtcag gagatcgaga ccatcctggt taacacagtg aacccctctc
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1681 cctccaggct tacagggcac tcttctatgc ccatcccaag tatagctgaa ccttccaagg
1741 gccagacttg gtgctaagta ccaagtacgc aaagattaat aaaacaatgt cctgtttcag
1801 ggagctcaaa gctgattcgg cagggcatgg tgtgtacatg aatgataacc acgtagggtt
1861 gcaggtttcc tagtgaggta agcacaaggc aagatgggaa acaaaggaag gaggggttca
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1981 ggaacaccca gctcagagag ggggttagac ctgcaggcta acacagacac agcccagaaa
2041 acccaggagc cgaggggggaa ggagaaaggt gcaagaaggg gaaacccagg tcctggtccc
2101 cttctctctg cttcctggca gcagaactca gacagaaccc ttaagccagt ctaagtctgg
2161 caggaccagt aagttctgag ttagctccat actagtttct agcaggctct ttctcacttc
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2281 cctgaccctg atccataggt cacacagcag gcatccacag ggtgggcgtg ggccctctca
2341 tccctccctc ccactcactt cacgctggct gggccccaag gtgtttgcac cccttgcagt
2401 gagtgacctt ctctagtgca gcaagctcag aacctgctgc cactggagtt gtcccattgc
2461 tgatgcagaa aggtgaagaa ctagcagaac actggaaatg ccctccatct gggtccatgg
2521 ctacttaatg ctccctggca ggcaggagga caggtgctat tccctgttgg gacagatgaa
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2761 ccagatatct gaagaagtcc tgatgtcact gccccggtcc ttccccaggt agagcaacac
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2881 acacacacac acacacacaa atccaagaca acactactaa ggcttctttg ggaaggggaa
2941 gtagggatag gtaagaggaa agtaagggac ctcctatcca gcctccatgg aatcctgact
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3181 tgtacaggtg atgtaacatc tctgtgcctc agtttgctca ctataaaata gagacggtag
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3301 cttacccact tcccccaagc acagttgggg tgggggacag ctgaagaggt ggaaacatgt
3361 gcctgagaat cctaatgaaa tcggggtaaa ggagcctgga acacatcctg tgaccccgcc
3421 tgtcctgtag gaagccagtc tctggaaagt aaaatggaag ggctgcttgg gaactttgag
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3841 attgaaaact aagtttatta gagaggttag agaaggagga gctctaagga gaaaaaatcc
3901 tgtgccggga aaccttgatt gtggcttttt aatgaatgaa gaggcctccc tgagcttaca
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4141 acccacttcc caggcaacct gcctaacatg cttcgagatc t
```

Claims

1. An adenoviral vector comprising:
   (a) a mammalian regulatory region; and
   (b) a reporter gene operatively linked to the mammalian regulatory region (a).

2. An adenoviral vector according to claim 1, wherein the mammalian regulatory region is a cytokine regulatory region.

3. An adenoviral vector according to claim 1 or claim 2, wherein the regulatory region is a promoter or enhancer.

4. An adenoviral vector according to claim 3, wherein the promoter is a TNFα promoter or an IL-10 promoter.

5. An adenoviral promoter according to claim 4, wherein the cytokine promoter is encompassed in -1173 to +130 of the human TNF gene.

6. An adenoviral vector according to any preceding claim, wherein the reporter gene is selected from luciferase, lacZ and chloramphenicol acetyltransferase (CAT).

7. An adenoviral vector according to any preceding claim additionally comprising a polyadenylation site operatively linked 3' to the reporter gene.

8. An adenoviral vector according to any preceding claim, additionally comprising 3' to the reporter gene a 3'-UTR (Untranslated Region).

9. A method of studying a mammalian regulatory region activity comprising inserting an adenoviral vector according to any preceding claim bearing the regulatory region into a cell and measuring the amount of reporter gene product produced in response to the regulatory region.

10. A method according to claim 9, additionally comprising exposing the adenoviral vector within the cell to one or more inhibitors or activators of the regulatory region.

11. A method according to claim 9 or claim 10, wherein the cell is selected from a monocytic cell, macrophage and a primary cell.

12. A method according to claim 11, wherein the primary cell is obtained from diseased tissue.

13. A method according to claim 12, wherein the diseased tissue is rheumatoid arthritic.

14. A cell comprising an adenoviral vector according to any one of claims 1 to 8.

15. A kit for use in a method according to claim 9, comprising an adenoviral vector according to any one of claims 1 to 8.

16. A method of screening a compound as an enhancer or an inhibitor of a mammalian regulatory region, comprising the use of an adenoviral vector according to any one of claims 1-8.

Figure 1

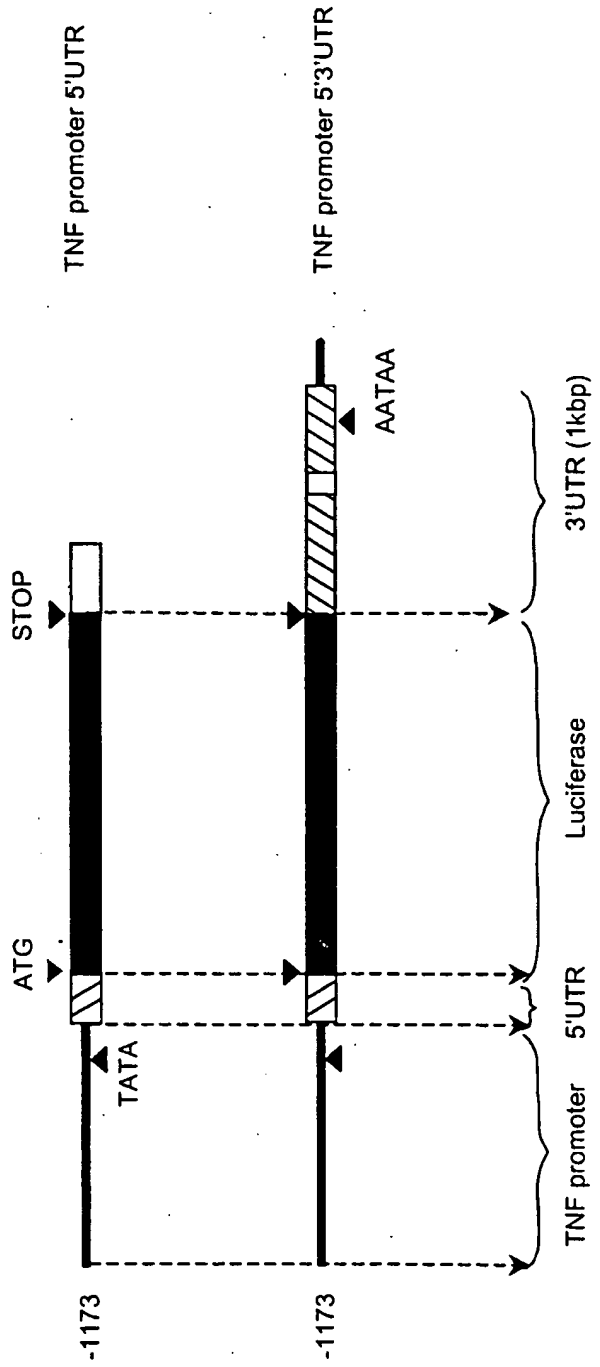

TNF promoter 5'UTR
TNF promoter 5'3'UTR
AATAA
3'UTR (1kbp)
STOP
Luciferase
ATG
5'UTR
TATA
TNF promoter
-1173
-1173

Figure 2

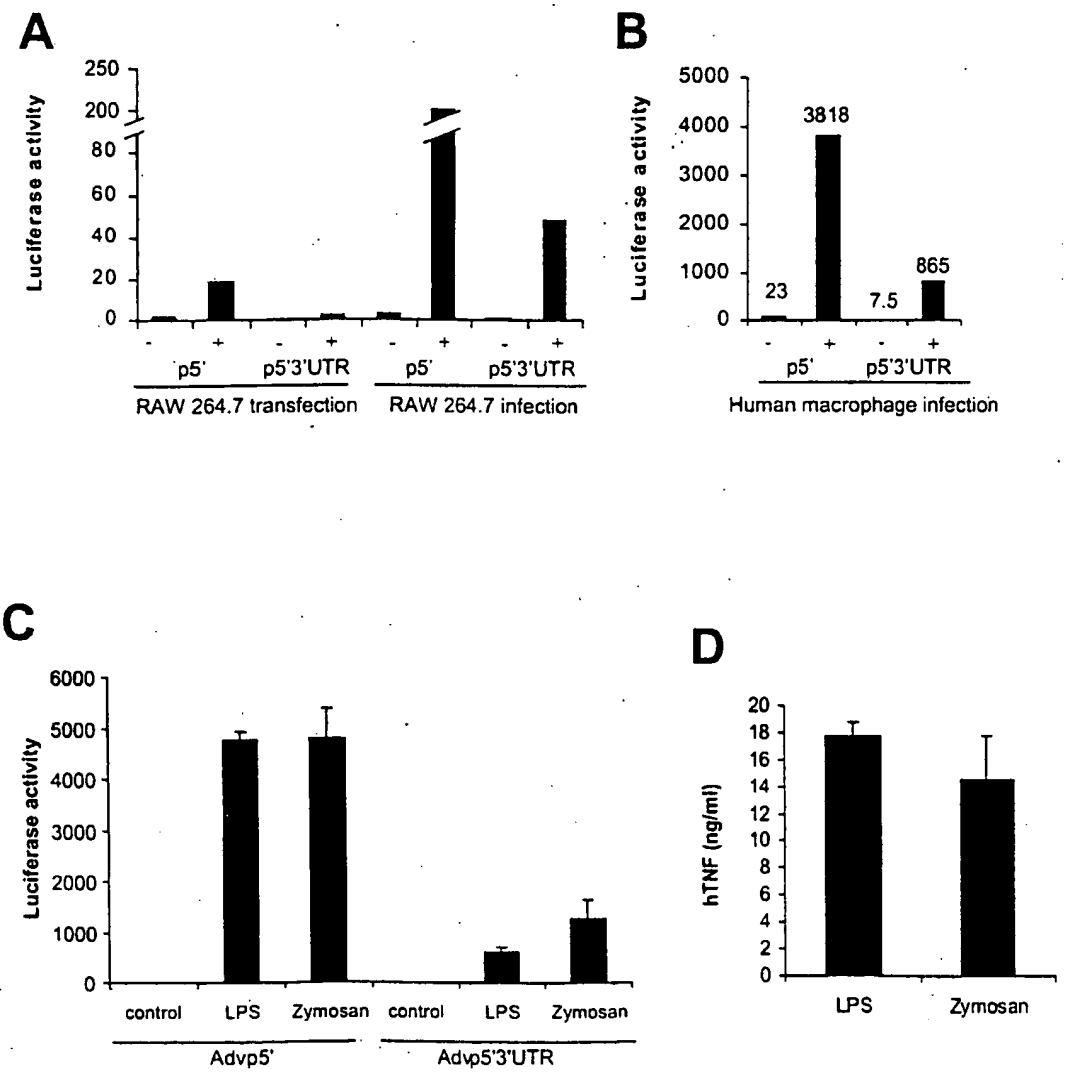
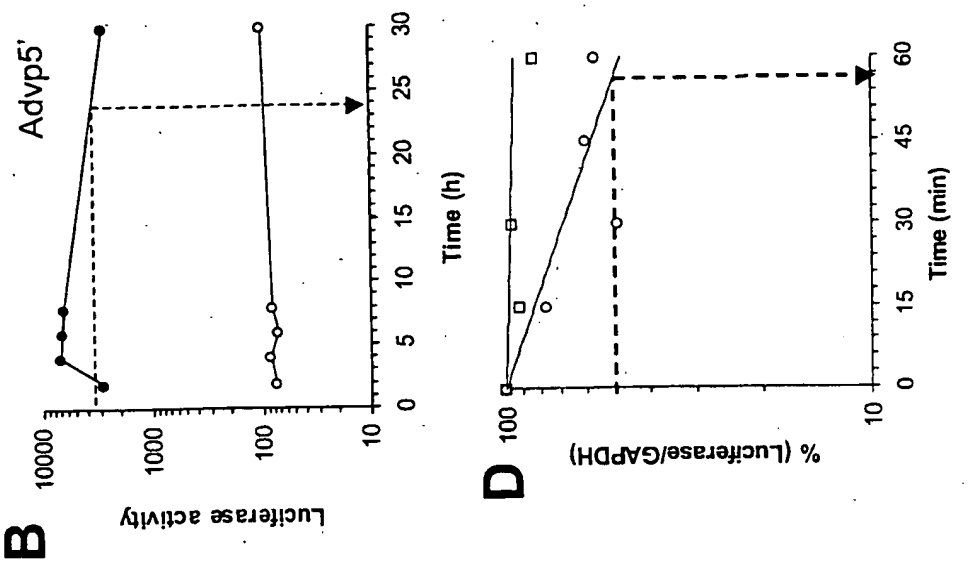

A
Luciferase activity
250
200
80
60
40
20
0
- + - + - + - +
p5' p5'3'UTR p5' p5'3'UTR
RAW 264.7 transfection
RAW 264.7 infection
B
Luciferase activity
5000
4000
3000
2000
1000
0
23
3818
7.5
865
- + - +
p5' p5'3'UTR
Human macrophage infection
C
Luciferase activity
6000
5000
4000
3000
2000
1000
0
control LPS Zymosan control LPS Zymosan
Advp5'
Advp5'3'UTR
D
hTNF (ng/ml)
20
18
16
14
12
10
8
6
4
2
0
LPS
Zymosan

Figure 3


B
Advp5'
Luciferase activity
10000
1000
100
10
0 5 10 15 20 25 30
Time (h)
D
% (Luciferase/GAPDH)
100
10
0 15 30 45 60
Time (min)

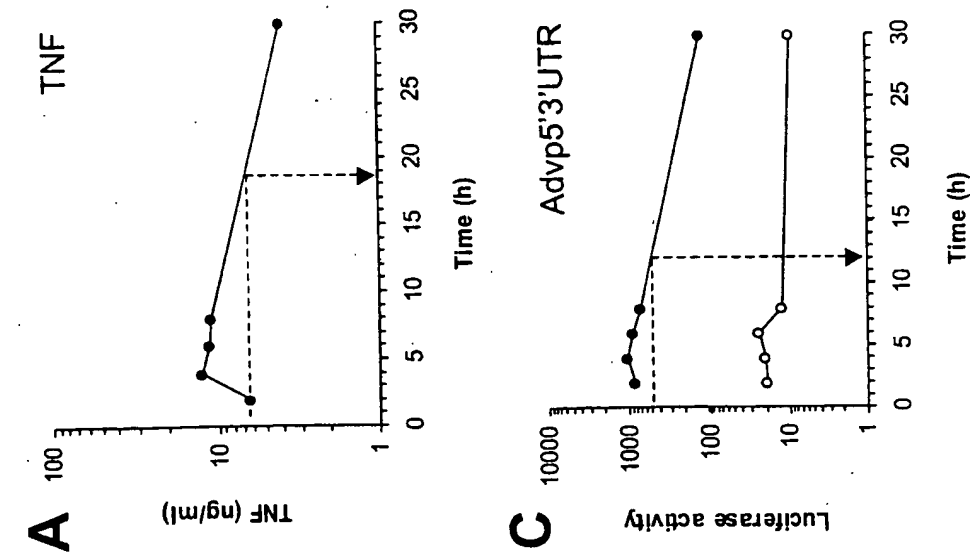
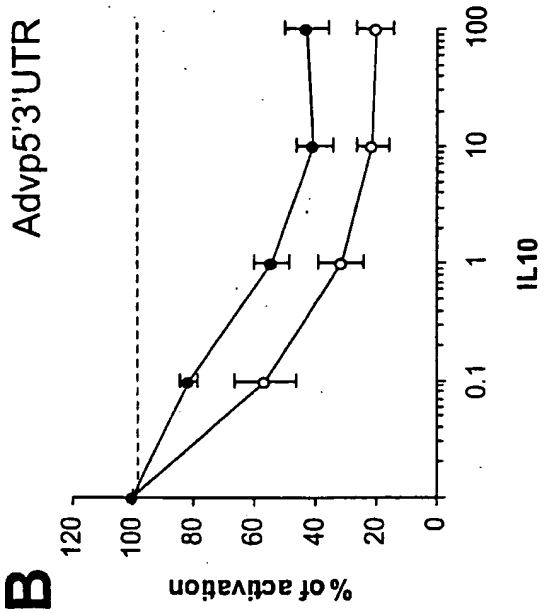

A
TNF
TNF (ng/ml)
100
10
1
0 5 10 15 20 25 30
Time (h)
C
Advp5'3'UTR
Luciferase activity
10000
1000
100
10
1
0 5 10 15 20 25 30
Time (h)

Figure 4


B
Advp5'3'UTR
% of activation
120
100
80
60
40
20
0
0.1
1
10
100
IL10

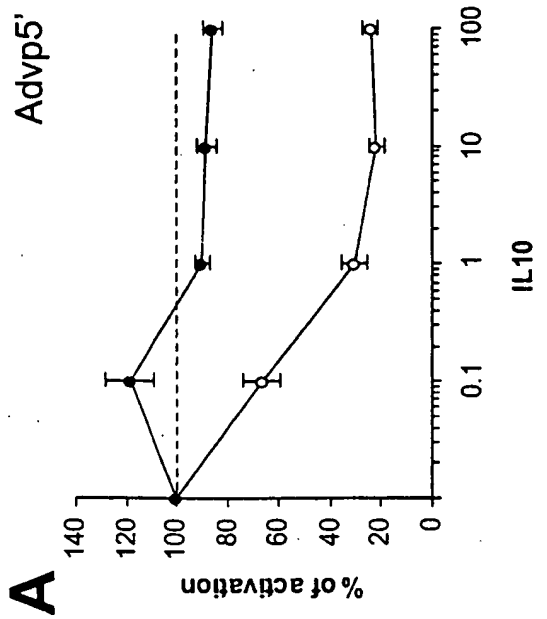
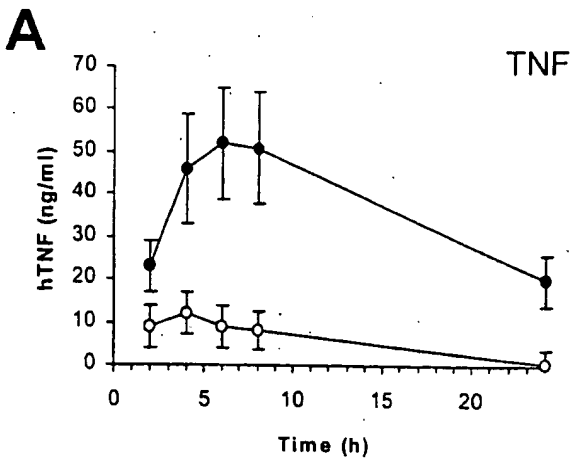
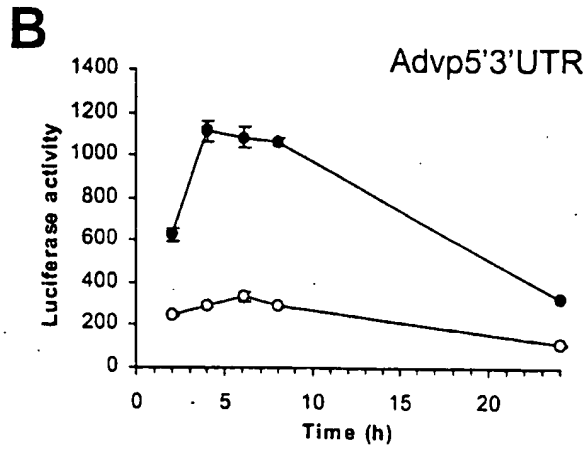

A
Advp5'
% of activation
140
120
100
80
60
40
20
0
0.1
1
10
100
IL10

Figure 5


A
TNF
hTNF (ng/ml)
70
60
50
40
30
20
10
0
0
5
10
15
20
Time (h)


B
Advp5'3'UTR
Luciferase activity
1400
1200
1000
800
600
400
200
0
0
5
10
15
20
Time (h)

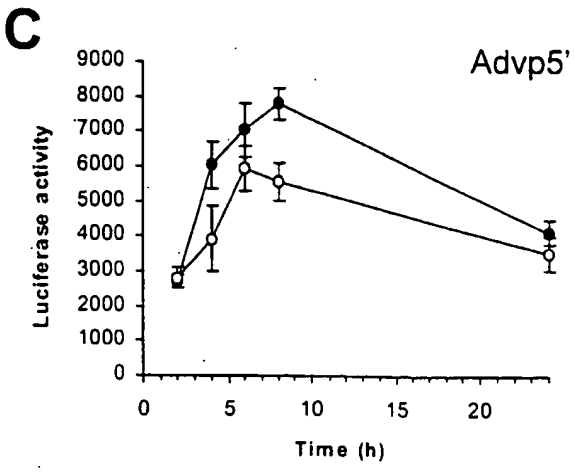
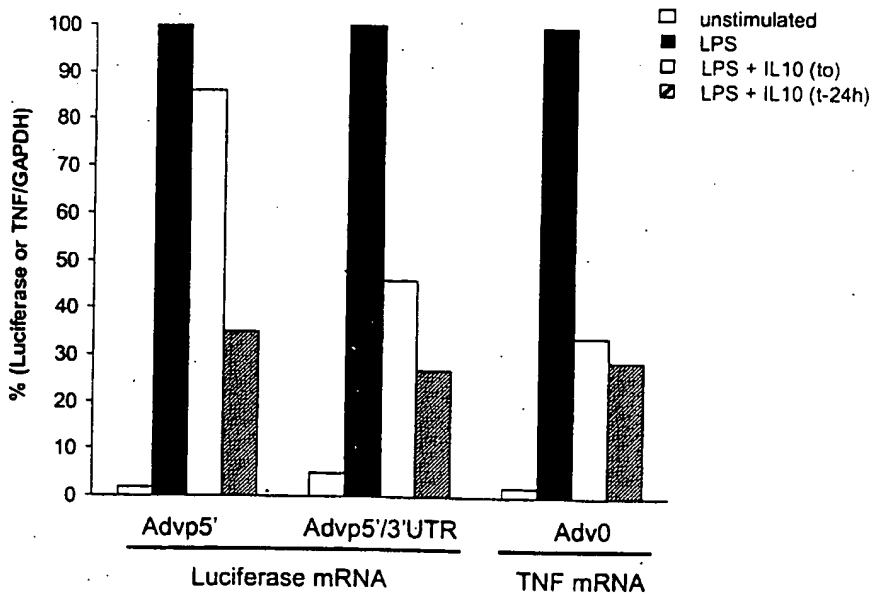

C
Advp5'
Luciferase activity
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
0
5
10
15
20
Time (h)

Figure 6


% (Luciferase or TNF/GAPDH)
100
90
80
70
60
50
40
30
20
10
0
unstimulated
LPS
LPS + IL10 (to)
LPS + IL10 (t-24h)
Advp5'
Advp5'/3'UTR
Adv0
Luciferase mRNA
TNF mRNA

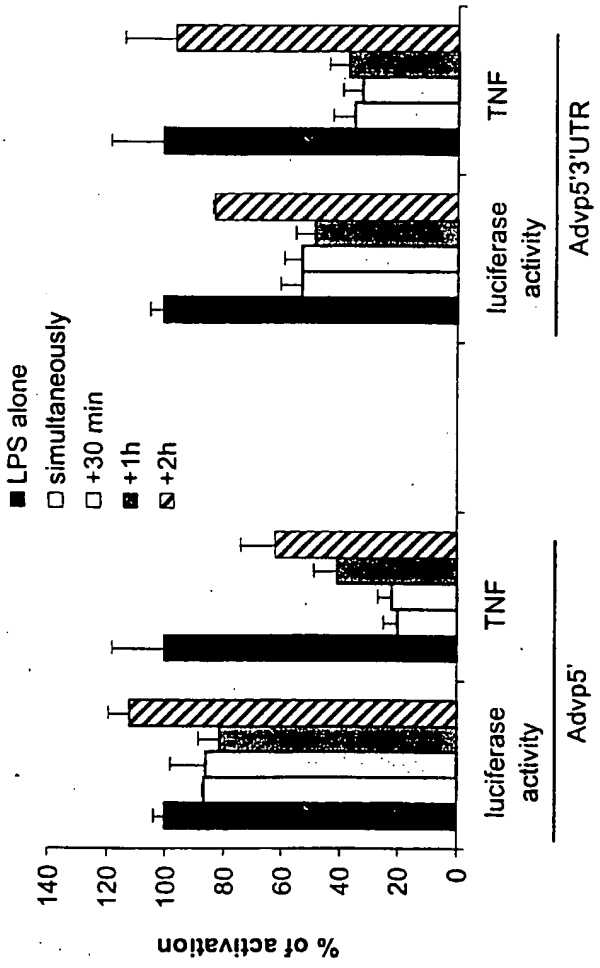

% of activation
140
120
100
80
60
40
20
0
LPS alone
simultaneously
+30 min
+1h
+2h
luciferase activity
TNF
Advp5'
luciferase activity
TNF
Advp5'3'UTR

Figure 7

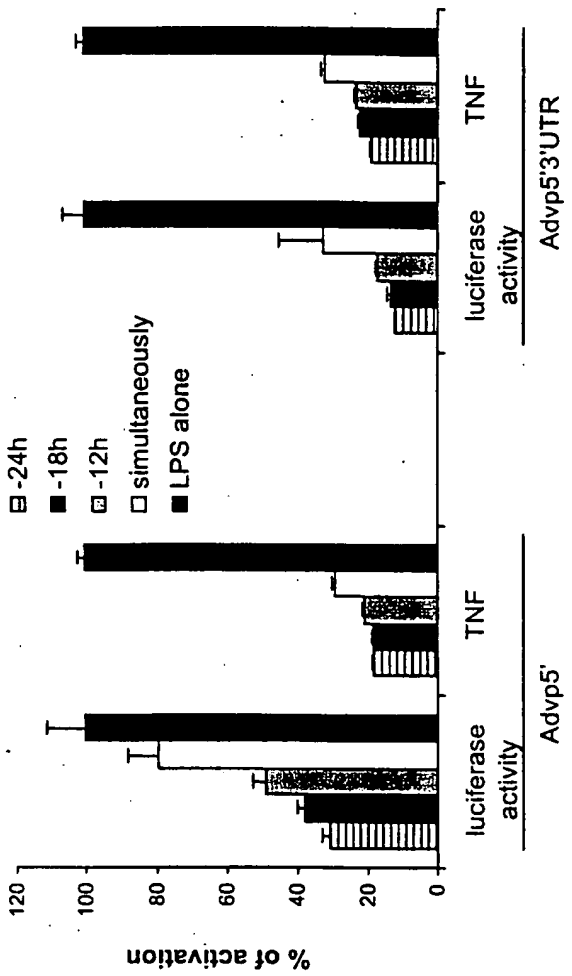

% of activation
120
100
80
60
40
20
0
-24h
-18h
-12h
simultaneously
LPS alone
luciferase activity
TNF
Advp5'
luciferase activity
TNF
Advp5'3'UTR

Figure 8

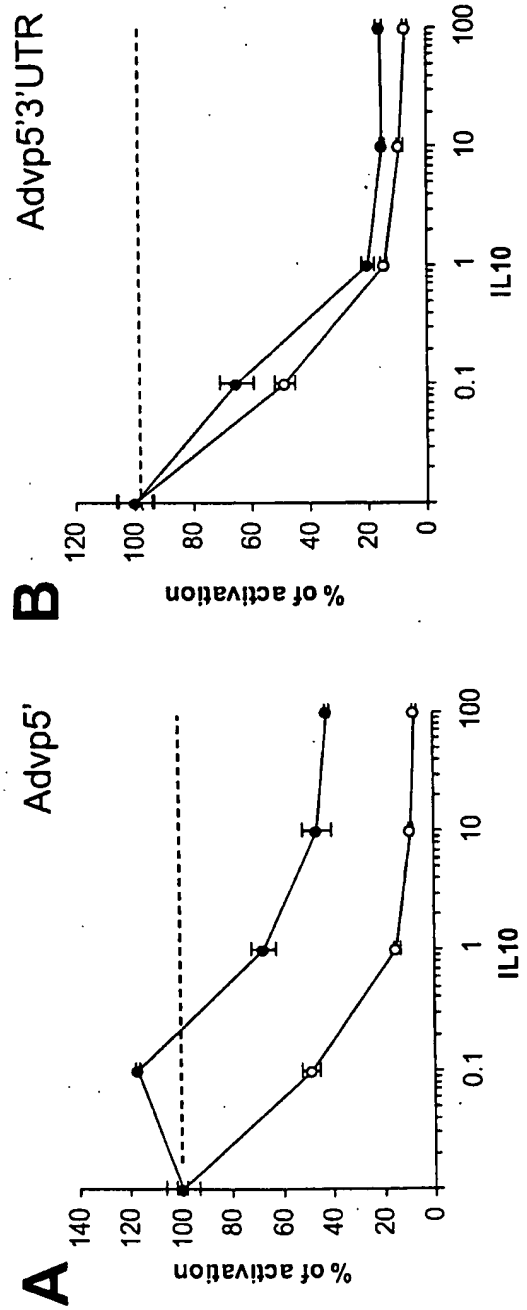
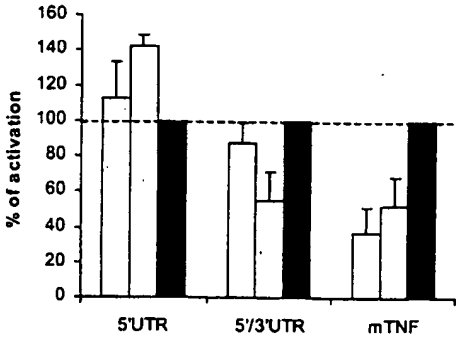

A
Advp5'
% of activation
140
120
100
80
60
40
20
0
0.1
1
10
100
IL10
B
Advp5'3'UTR
% of activation
120
100
80
60
40
20
0
0.1
1
10
100
IL10

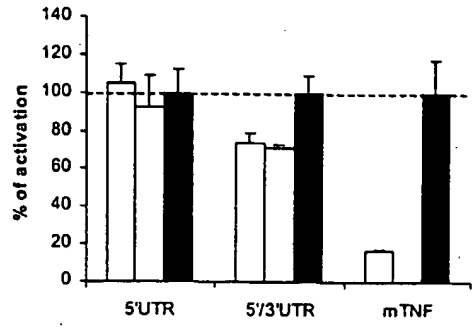
Figure 9

Figure 10

RAW 264.7 cells



Murine primary peritoneal macrophage



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(74) Agents: **ELSY, David** et al.: Withers & Rogers, Goldings House, 2 Hays Lane, London SE1 2HW (GB).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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(88) Date of publication of the international search report:
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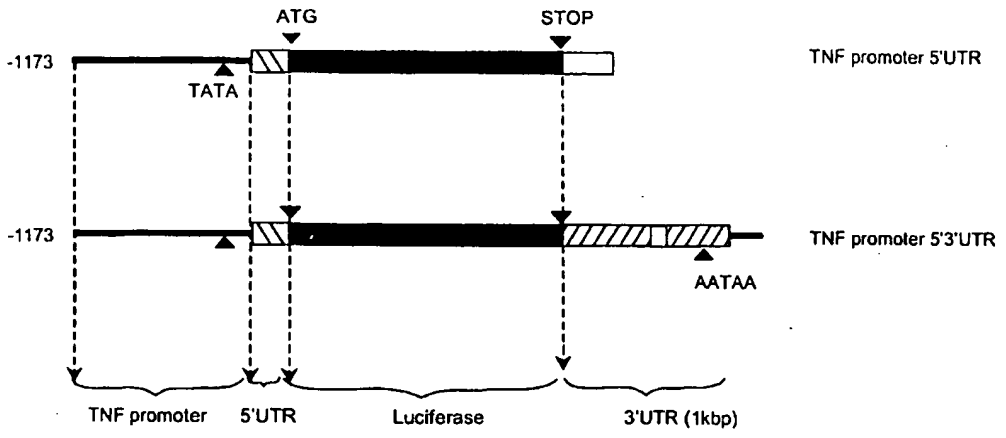
For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: ADENOVIRAL VECTOR WITH A REPORTER GENE LINKED TO A REGULATORY REGION



(57) Abstract: The invention relates to adenoviral vectors comprising a mammalian regulatory region such as a cytokine regulatory region and a reporter gene operatively linked to the cytokine promoter. Preferably, the cytokine promoter is a TNFα promoter or an IL-10 promoter. The application also disclosesmethods of studying cytokine promoter activity and to cells containing the adenoviral vectors.

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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/GB 00/03645

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/86 C12N15/24 C12N15/28

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, PAJ, EPO-Internal, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	UDALOVA ET AL: "Complex NF-kB interactions at the distal tumor necrosis factor promoter region in human monocytes" JOURNAL OF BIOLOGICAL CHEMISTRY , vol. 273, 1998, pages 21178-21186, XP002162886 cited in the application * Page 21178 (Introduction); page 21179, plasmid (hTNF promoter sequence); page 21184 (Discussion) * ---	1-16
Y	BONDESON ET AL: "Adenoviral transfer of the IkB gene inhibits the induction of proinflammatory cytokines" ARTHRITIS & RHEUMATISM, vol. 40, September 1997 (1997-09), page S322 XP000981222 * Abstract * ---	1-16

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 March 2001	30/03/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Korsner, S-E

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INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/GB 00/03645

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	ROBERT ET AL.: "An adenoviral vector-based system to study neuronal gene expression: Analysis of the rat tyrosine hydroxylase promoter in cultured neurons" JOURNAL OF NEUROCHEMISTRY, vol. 68, 1997, pages 2152-2160, XP000989957 * Page 2152 (column 2) - page 2153 (column 1); figure 1; pages 2157-2159 (Discussion) * ---	1-16
A	KAWASAKI ET AL.: "Selection of the best target site for ribozyme-mediated cleavage within a fusion gene for adenovirus E1A-associated 300 kDa protein (p300) and luciferase" NUCLEIC ACIDS RESEARCH, vol. 24, 1996, pages 3010-3016, XP002162887 * Page 3013, column 1 * ---	1-16
A	WO 99 09045 A (SOMAGENICS) 25 February 1999 (1999-02-25) * Example I(C); Figure 4 * ---	1-16
Y,P	NETTELBECK ET AL.: "GENE THERAPY / Designer promoters for tumour targeting" TIG, vol. 16, April 2000 (2000-04), pages 174-181, XP002162888 * Tables & figures * -----	1-16

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter onal Application No
PCT/GB 00/03645

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9909045 A	25-02-1999	AU 9199998 A	08-03-1999
		EP 1019429 A	19-07-2000